T cell cytokine receptor (TCCR)/WSX-1 is required for efficient development of Th1 response in cutaneous leishmaniasis caused by *Leishmania mexicana*

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Abstract

Lesion development and *Leishmania mexciana* specific immune responses were measured in T cell cytokine receptor knockout (TCCR -/-) mice and compared to similarly infected wildtype (TCCR +/+) control mice. In comparison to TCCR +/+ mice, TCCR -/- mice developed larger ulcerating lesions containing higher parasite loads following inoculation with 1000 *L. mexicana* amastigotes into ear dermis. These differences became significant nine weeks after infection. Previous studies have found that the interleukin 27 (IL-27)/TCCR signaling pathway mediates susceptibility to visceral leishmaniasis caused by *Leishmania donovani*, but plays a minor role in determining outcome of cutaneous leishmaniasis resulting from *Leishmania. major* infection. However the results of the present study provide new observations related to IL-27 signaling and cutaneous leishmaniasis caused by *L. mexicana*. Specific immunologic responses of *L. mexicana* infected TCCR -/- and TCCR +/+ mice were therefore examined both *in vivo* and *in vitro*. In comparison to TCCR +/+ infected with *L. mexicana*, TCCR -/- mice generated minimal levels of type 1 (Th1)-associated immunoglobulin G2a (IgG2a) antibodies. Additionally TCCR -/- mice up-regulated parasite specific immunoglobulin G1 (IgG1) antibodies as infection progressed indicating a dominant type-2 (Th2) response in TCCR -/- mice. Furthermore, draining lymph node cells and splenocytes harvested from *L. mexicana* infected TCCR -/- mice and stimulated in vitro with *L. mexicana* (Lm-Ag) antigen produced substantially less IFN-γ, but more Il-4 and Il-10 as compared to TCCR +/+ counterparts. This again indicates a predominately Th2-influenced immune response rather than Th1 response in TCCR -/- mice infected with *L. mexicana*. These results demonstrate that whereas the IL-27/TCCR signaling pathway is dispensable against infection with *L. major*, it is required to mount a Th1 response and control cutaneous leishmaniasis caused by *L. mexicana*.

Keywords

IL-27, TCCR, WSX-1, Leishmania, L. Mexicana, L. Major, Th1 immunity, cytokines

Introduction

Leishmania are obligate intracellular pathogens which infect host macrophages and depending on the species, result in cutaneous, mucocutaneous, or visceral forms of disease. Cutaneous manifestations of disease are caused by the old world species, *Leishmania major* and the new world species, *Leishmania mexicana*. Mice on a resistant background like C57BL/6 are able to resolve lesions resulting from *L. major*; however following infection with *L. mexicana*, they often develop chronic non-healing lesions (14). In order to control infection the host must mount a strong type 1 (Th1) immune response and produce sufficient levels of gamma interferon (IFN-γ) for macrophage activation and parasite killing (1). Macrophage-derived interleukin-12 (IL-12) is a central cytokine for Th1 immunity and IFN-γ production, and thus is necessary to combat cutaneous leishmaniasis (1). Increasing evidence has shown that host immune mechanisms against *L. major* and *L. mexicana* are different (7). For example, while susceptibility to *L. major* is caused by a dominant type 2 (Th2) response, susceptibility to *L. mexicana* has been attributed to a weak Th1 response due to lack of IL-12 production (12).

In addition to IL-12, newly discovered cytokines have emerged which also play important roles during Th1 immunity. IL-27, structurally similar to IL-12, is a new member of the IL-6/IL-12 family of cytokines. IL-27 is a heterodimeric cytokine composed of Epstein-Barr virus-induced gene 3 (EBI3) and p28 subunits and mediates its effects via a heterodimeric receptor composed of TCCR also known as WSX-1, a class I cytokine receptor homologous to IL-12 receptor ß2 chain (IL-12 R ß2), and glycoprotein 130 (gp130) (11). While various cytokines employ gp130, which is expressed on both immune and non-immune cells, TCCR is specific for IL-27 and restricted to cells of the immune system such as monocytes, Langerhan's cells, dendritic cells, natural killer cells, T-cells, and B-cells (14).

The IL-27/TCCR pathway is implicated in the early establishment of the Th1 response and has been found to mediate resistance against certain intracellular pathogens, like *Listera monocytogenes* and *L. major* (14). Previous studies with *L. major* revealed that TCCR -/- mice were more susceptible to infection and produced less IFN-γ than wild type counterparts (18). However, the requirement of IL-27/TCCR signaling was found to

be transient and limited to early stages of infection because TCCR -/- mice were eventually able to mount an effective Th1 response and resolve infection (17). Additional studies with *L. major* revealed that the requirement for TCCR signaling for Th1 development was required in a cytokine milieu containing IL-4 (2). Therefore, the protective nature of IL-27 during infection with *L. major* was attributed to its ability to inhibit Th2 cytokine production by counteracting the polarizing nature of IL-4. Moreover, IL-27 signaling was also shown to down-regulate IL-4 expression and prime naïve T cells toward a Th1 immune response by increasing responsiveness to IL-12 (8; 9; 16).

However, the precise function of IL-27 during Th1 immunity is complicated because there have also been anti-inflammatory functions associated with this cytokine (6). For instance, during visceral leishmaniasis, induced by *Leishmania donovani*, TCCR -/- mounted a robust Th1 responses generating excessive amounts of IFN-γ and tumor necrosis factor alpha (TNF-α) causing severe hepatic inflammation in TCCR -/- (14). In contrast to cutaneous leishmaniasis IL-27 signaling was required to down regulate pro-inflammatory T-cell cytokine production during visceral leishmaniasis (14). In addition IL-27 signaling is not necessary for Th1 development, but required to limit T cell responses during infection with other intracellular pathogens like *Toxoplasma gondii* and *Trypanosoma cruzi* (17). Thus, the requirement of IL-27 signaling to induce Th1 development appears to depend on the nature of pathogen and prevailing cytokine microenvironment (6).

IL-27/TCCR signaling has been well characterized during infection caused by *L. major* and *L. donovani*, however little is known about its function during infection with *L. mexicana*. In this study, we defined the role of IL-27 signaling against *L. mexicana* by infecting C57BL/6 mice deficient in TCCR and monitoring disease progression, parasite loads, and cytokine production. TCCR -/ - mice developed larger lesions and contained higher parasite loads then TCCR +/+ mice counterparts. In addition, *L. mexicana* antigen (Lm-Ag) stimulated regional draining lymph node cells and splenocytes from TCCR -/- mice produced lower levels of Th1 cytokines, IFN-γ and IL-12, and higher levels of Th2 cytokines, IL-4 and IL-10, compared to TCCR +/+ C57BL/6 mice. These results demonstrate that TCCR -/- failed to mount an effective Th1 immune

response against, and consequently were unable to control infection. Therefore, the IL-27/TCCR signaling pathway is required in development of Th1 immunity which mediates resistance to *L. mexicana*.

Materials and Methods

Animals

TCCR -/- C57BL6 mice were generously donated by Christ Hunter. Age and sex matched wild-type C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). The animals were infected at 8-10 weeks of age and were maintained in the specific pathogen free (SPF) facilities at the Ohio State University animal facilities in accordance with institutional guidelines. In each experiment four to five mice per group were included.

Parasites and infection protocol

Leishmania mexicana parasites (MNYC/BZ/62/M379) were maintained by serial passage of amastigotes inoculated s.c. into the shaven rumps of 129SvE mice. Amastigotes isolated from the lesions of infected mice were grown *in vitro*. Mice were infected intra-dermally by inoculating 10^3 *L. mexicana* stationary phase metacyclic promastigotes in a volume of 10 µL into ear dermis using a 30-guage needle. Metacyclic parasites were isolated using a peanut agglutination procedure. Lesion growth was monitored at weekly intervals by measuring the thickness of the infected ear using a dial-gauge micrometer and was expressed as the increase in thickness of the infected ear compared with the uninfected ear.

Quantification of parasite burdens

Parasite burdens were quantified weeks after infection at 3 week intervals. Infected mice were euthanized and relative parasite burdens in the infected ears were determined by limiting dilution analysis. The two sheets of infected ears were separated, placed dermal side up, and ground on a nylon cell strainer in RPMI 1640 medium supplemented with 2mM L-glutamine, 10% fetal bovine serum (heat inactivated), 100 U mL penicillin, 100 µg ml streptomycin, and 0.5 mM B-mercaptoethanol (GIBCO, BRL Grand Island, NY). Amastigotes isolated from lesions of infected mice were grown in vitro. Limiting dilution analysis with parasite suspensions was carried out as described previously. The results were expressed as reciprocal log parasite titers.

Cytokine assays

At 3, 6, 9, and 12 weeks post-infection the retromaxillar draining lymph nodes and spleens were removed in sterile conditions. Single-cell suspensions were prepared by gentle teasing in RPMI 1640 medium supplemented with 2mM L-glutamine, 10% fetal bovine serum (heat inactivated), 100 U ml penicillin, 100 µg ml streptomycin, and 0.5 mM B-mercaptoethanol (GIBCO, BRL Grand Island, NY). Viable cells were counted by trypan blue exclusion and adjusted to 3×10^6 cells ml for lymph nodes and 5×106 cells ml for spleens in the same medium. Aliquots of 100 µl were placed into 96-well flat bottom culture plates (Costar, Cambridge, MA, USA) and stimulated with 20 µg/ml of Lm-Ag prepared by seven cycles of repeated freezing in liquid nitrogen (-70°C) and thawing. Supernatants were collected from parallel cultures after 72 hours after incubation for enzyme-linked immunosorbent assay (ELISA) quantification of cytokine production. Cytokine levels (IL-12, IFN-y, IL-4, and IL-10) in supernatants were measured using sandwich ELISA according to the manufacturer's instructions (BD PharMingen, San Diego, CA, USA). Antibody clones used were as follows: IL-12, clone C15.6 (capture) and clone C17.8 (detection); IFN-γ, clone R4-6A2 (capture) and clone XMG1.2 (detection); IL-4, clone BVD4-1D11 (capture) and clone BVD6-24G2 (detection); and IL-10, clone JES5-2A5 (capture) and clone SXC-1 (detection).

Antibody ELISA

Peripheral blood was obtained from infected mice by tail snapping at various 2 week intervals post-infection. Plasma samples were obtained following centrifugation at 200 g and were used to analyze the levels of L. mexicana-specific Th2-associated immunoglobulin G1 (IgG1) and Th1-associated immunoglobulin G2a (IgG2a) antibodies by ELISA.

Statistical Analysis

All the data showed in this study were obtained from two or three different experiments unless specified otherwise. Comparisons between TCCR +/+ and TCCR -/- mice were made using Student's unpaired t-test. $P < 0.05$ was considered significant. The statistical significance of the sera titers was determined by using Mann-Whitney U prime test.

Results

Course of L. mexicana ear infection in TCCR -/- mice and TCCR +/+ counterparts

Following inoculation with 1000 metacyclic *L. mexicana* promastigotes into the ear dermis, TCCR -/- mice and TCCR +/+ mice counterparts were monitored weekly for progression of the disease. TCCR -/- mice generated larger ulcerating ear lesions than TCCR +/+ mice (Fig. 1). The progression of ear lesion size was significantly enhanced by 9 weeks post infection in TCCR -/- in comparison to TCCR +/+ mice (Fig. 1). Contrary to the results seen with L. major TCCR -/- mice continued to display enhanced ear lesion growth.

Parasite burdens of L. mexicana in ear lesions of TCCR -/- and TCCR +/+ mice

At three week intervals post inoculation infected ears were removed from TCCR -/- and TCCR +/+ mice and parasite loads were quantified using limiting dilution analysis. In comparison to TCCR +/+ mice, lesions from TCCR -/- mice contained more parasites (Fig. 2). TCCR -/- mice maintained higher numbers of parasites at the site of the lesion, which was significant at 9 and 12 weeks post infection (Fig. 2).

Antibody production in TCCR +/+ and TCCR -/- following infection with L. mexicana

Following infection with *L. mexicana*, mice were bled by tail snipping and sera were obtained. Serum levels of L. mexicana-specific IgG1 and IgG2a antibodies were measured in plasma samples using ELISA. In comparison to TCCR +/+ mice, TCCR -/- mice maintained low levels of Th1-associated IgG2a and up regulated Th2-associated IgG1 (Fig.3A, B).

Kinetics of in vitro cytokine production by LmAg-stimulated regional draining lymph node cells from TCCR -/- and TCCR -/- mice after infection with L. mexicana

The production of Th1- associated cytokines, IL-12 and IFN-γ, and Th2 – associated cytokines, IL-4 and IL-10 by lymph node cells from infected mice were analyzed. Cells from the regional draining lymph nodes were isolated and stimulated with *L. Mexicana* antigen (Lm-Ag) at 3, 6, 9, and 12 weeks post-infection and cytokine production in culture supernatants were quantified using ELISA. In comparison to

TCCR +/+ mice, Lm-Ag stimulated lymph node cells from TCCR -/- mice generated robust levels of Th2 cytokines while maintaining minimal amounts of IFN-γ. Culture supernatants from both groups contained comparable levels of IL-12 throughout infection with TCCR -/- mice displaying less IL-12 at 3 and 9 weeks post infection as compared to TCCR +/+ mice (Fig. 4B). Additionally, at 9 and 12 weeks post infection Lm-Ag stimulated lymph node cells from TCCR -/- mice produced dramatically less IFN-y as compared to TCCR +/+ counterparts (Fig 4A). While both groups appeared to up regulate IL-4, Lm-Ag stimulated lymph node cells harvested from TCCR -/- mice produced significantly higher levels, which was pronounced at 9 and 12 weeks post infection (Fig 4C). In comparison to TCCR +/+ mice, IL-10 production also increased considerably in Lm-Ag stimulated lymph node cells from TCCR -/- mice at 12 weeks post-infection (Fig. 4D).

Kinetics of in vitro cytokine production by LmAg-stimulated splenocytes from TCCR -/- and TCCR -/- mice after infection with L. mexicana

Splenocytes from infected TCCR -/- and TCCR +/+ mice were also isolated and stimulated with Lm-Ag at 3, 6, 9, and 12 weeks post-infection. The production of Th1-associated cytokines and Th2 - associated cytokines was determined in culture supernatants using ELISA. Similar to Lm-Ag stimulated lymph node cells, splenocytes from TCCR -/- mice generated significantly higher levels of Th2 cytokines, IL-4 and IL-10, at 9 and 12 weeks post-infection (Fig 5C, D). IL-12 production by Lm-Ag stimulated splenocytes from TCCR -/- and TCCR +/+ mice was not considerably different (Fig 5B). However in comparison to TCCR +/+ mice, culture supernatants from TCCR -/- mice contained minimal levels of IFN-γ throughout infection similar to Lm-Ag stimulated lymph node cells (Fig 5A).

Discussion

The results of the present study indicate that the IL-27/TCCR signaling pathway is required for effective Th1 differentiation. Thus unlike *L. major* and *L. donovani*, IL-27 plays a significant role in mediating resistance to cutaneous leishmaniasis induced by *L. mexicana*.

The protective nature of IL-27/TCCR signaling during cutaneous leishmaniasis was first demonstrated with *L. major*. TCCR -/- mice infected with *L. major* were initially more susceptible to infection because they mounted a Th2 type of immune response and produced less IFN-γ as compared to wildtype littermates (18). However, this lack of IFN-γ was limited to the early stages of infection and *L major* infected TCCR -/- mice eventually produced sufficient levels and resolved infection (18). Additional studies revealed that IL-27/TCCR signaling was not essential for resistance to *L. major* in the absence of IL-4 (2). Therefore, while IL-27 is capable of inducing Th1 development during infection with *L. major* it primarily functions in limiting Th2 responses (2).

Following infection with *L. mexicana*, TCCR -/- mice displayed greater susceptibility due to an exaggerated Th2 response and insufficient production of IFN-γ. Relative to TCCR +/+ mice, infected TCCR -/- mice displayed enhanced levels of Th2-associated antibody and cytokine production. Thus, like *L. major*, IL-27/TCCR signaling pathway is also required to suppress Th2 responses against *L. mexicana* infection. Supporting this, in *vitro* studies have shown that IL-27 can inhibit Th2 development by suppressing expression of GATA-3 and subsequent IL-4 production (2).

TCCR -/- mice infected with *L. mexicana* also sustained minimal levels of IFN-γ throughout the course of disease. So, in addition to inhibiting Th2 responses, the IL-27/TCCR signaling pathway is also required for Th1 differentiation and optimal IFN-γ production in order to control cutaneous leishmaniasis caused by *L. mexicana*.

Interestingly both TCCR -/- and TCCR +/+ mice maintained comparable levels of IL-12 after infection with *L. mexicana*, however TCCR -/- mice were unable to mount a strong Th1 response and produced significantly less IFN-γ in comparison to TCCR +/+ mice. Infected TCCR-/- mice displayed a marked lack of responsiveness to IL-12. Therefore IL-27 may be required to limit severity of *L. mexicana* disease by priming T-cells for Th1 differentiation and enhancing their sensitivity to IL-12. The role of IL-27 in

the development of Th1 responses has been further characterized through *in vitro* studies, which revealed that IL-27 signaling can up regulate IL-12R β2 chain expression on T cells through a signal transducer and activator of transcription factor 1 (STAT-1) dependent mechanism (9; 16). Furthermore these studied showed that IL-27 alone cannot generate Th1 immunity, but can increase IFN-γ production indirectly by increasing T cell responsiveness to IL-12 (16).

However, the IL-27/TCCR signaling pathway is not always required for IFN-γ production. For example, IL-27 is more important in limiting Th1 responses to *L. donovani*, *T. gondii*, and *T. cruzi* (5; 14). Consequently IL-27 also has powerful anti-inflammatory properties to mediate inflammation (5). Recent studies have tried to resolve these antagonistic roles of IL-27 during cellular immunity.

According to previous works and the present study, the differential requirement of IL-27 during Th1 immunity largely depends on the pathogen and resulting cytokine milieu. Thus the requirement of IL-27 signaling to induce Th1 immunity during *L. mexicana*, but not *L. major* or *L. donovani*, can be attributed to the different cytokine microenvironments resulting from each species. For example, unlike *L. major*, susceptibility to *L. mexicana* is independent of IL-4 and rather due to a weak Th1 immune response dependent on IL-12 (7; 8). Further studies have also shown that IL-12 production is significantly reduced in mice infected with *L. mexicana* (12). The IL-27/TCCR signaling pathway may be required to induce Th1 differentiation in these conditions where IL-12 concentrations are minimal**.** In support of this a recent *in vitro* study found that in the absence, or under low concentrations of IL-12, IL-27 was required to efficiently induce Th1 differentiation through an alternative mechanism independent of IFN-γ and STAT-4 (10). Consequently IL-27 signaling may be necessary for efficient Th1 development against *L. mexicana,* but not *L. major*, because of decreased IL-12 production.

Although IL-27 is not necessary for protective immunity for *L. donovani*, or *L. major*, our study indicates that IL-27/TCCR signaling is important in mediating resistance to *L. mexicana*. The differential role of IL-27 can be attributed to different cytokine microenvironments. The environment induced by *L. mexicana* is not highly Th1 polarizing, which may explain the requirement for IL-27 to develop protective Th1

immunity against *L. mexicana*, but not *L. major* or *L. donovani*. Moreover, since IL-27 can suppress Th2 cytokine levels, it is difficult to distinguish whether IFN-γ production is directly influenced by IL-27 signaling or a result of down regulation of IL-4. Additional studies may elucidate whether this signaling pathway plays a direct role in Th1 induction or if it secondary to its role in limiting Th2 responses in order to control *L. mexicana* infection.

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Figure 1: Course of *L. mexicana* ear infection in TCCR -/- mice and TCCR +/+ counterparts. Ear lesion growth was monitored by measuring the thickness of the infected ear using a dial-gauge micrometer at weekly intervals and was expressed as the increase in thickness f the infected ear compared with the uninfected ear. Data are expressed as mean lesion size (millimeters) ± SE. The data are the resultant means of two independent experiments (n=5 mice per group). Asterisks indicate statistically significant ($P < 0.05$) differences between the groups.

Figure 2: Parasite burdens of *L. mexicana* in ear lesions of TCCR -/- and TCCR +/+ mice. Parasite loads in ear lesions of TCCR -/- and TCCR +/+ mice were determined 3, 6, 9, and 12 weeks post-infection by limiting dilution analysis and data are expressed as mean log dilution ± SE. The data shown for 21 and 63 days post-infection are means of 2 independent experiments (n=5 mice per group). The data shown for 84 days after infection are from one experiment. Asterisks indicate statistically significant ($P < 0.05$) differences between the groups.

Figure 3: Antibody production in TCCR +/+ and TCCR -/- following infection with *L. mexicana.* Serum samples were obtained from mice at different time points by tail snipping and Th1-associated and Th2-associated antibody titers were measured using ELISA. The data are the means from one experiment, but representative of two independent experiments (n=5-8 samples). Data expressed as reciprocal endpoint titer ± SE. ND, no antibody detected at the 1: 100 dilution. (* denotes $P < 0.05$)

Figure 4: Kinetics of (A) IFN-γ, (B) IL-12, (C) IL-4, (D) IL-10 production by Lm-Ag-stimulated regional draining lymph node cells from TCCR -/- and TCCR -/- mice infected with *L. mexicana*. The data shown for 3, 6, 9 weeks after infection are means of 2 independent experiments for each time point (n = 4-5 mice). The data shown for 12 weeks post-infection are from one experiment (n=5 mice). Data expressed as means ± SE. Asterisks indicate statistically significant ($P < 0.05$) differences between the groups.

Figure 5: Kinetics of (A) IFN-γ, (B) IL-12, (C) IL-4, (D) IL-10 production by LmAg-stimulated splenocytes from TCCR -/- and TCCR -/- mice infected with *L. mexicana*. The data shown for 3, 6, 9 weeks after infection are means of 2 independent experiments for each time point (n = 4-5 mice). The data shown for 12 weeks post-infection are from one experiment (n=5 mice). Data expressed as means ± SE. Asterisks indicate statistically significant ($P < 0.05$) differences between the groups.

Figure 1

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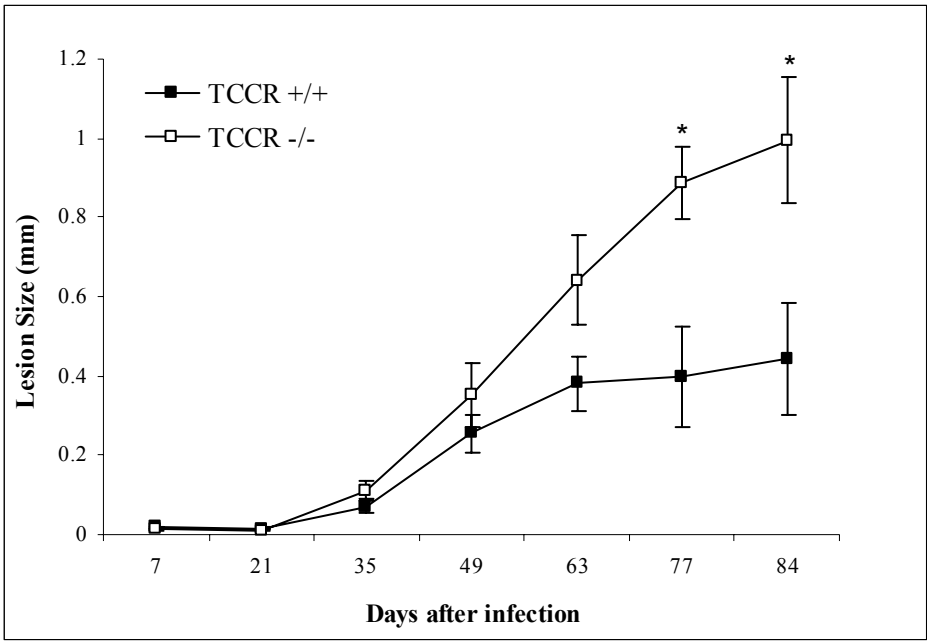

Figure 2

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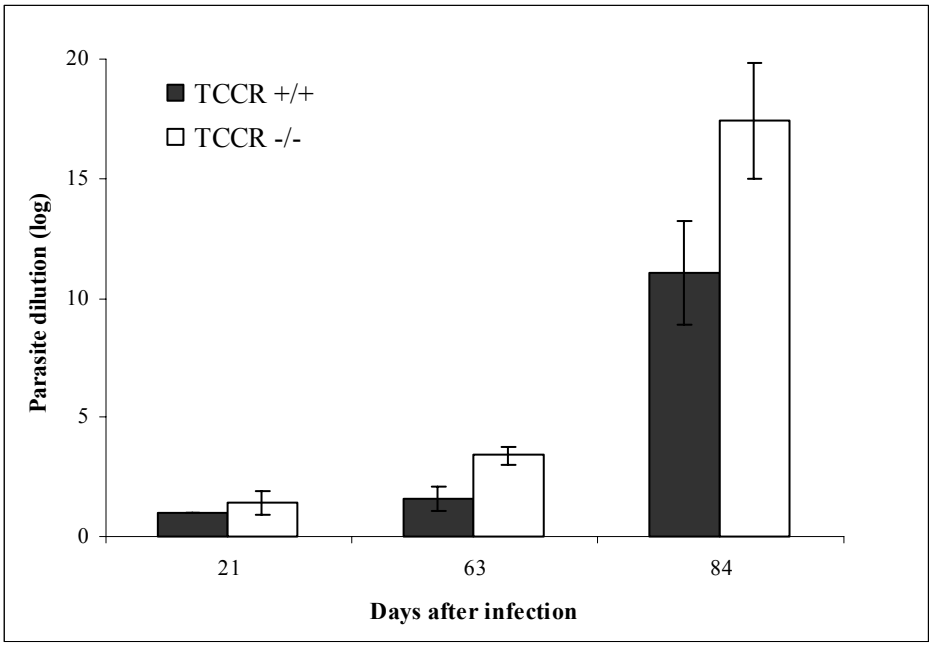

Figure 3

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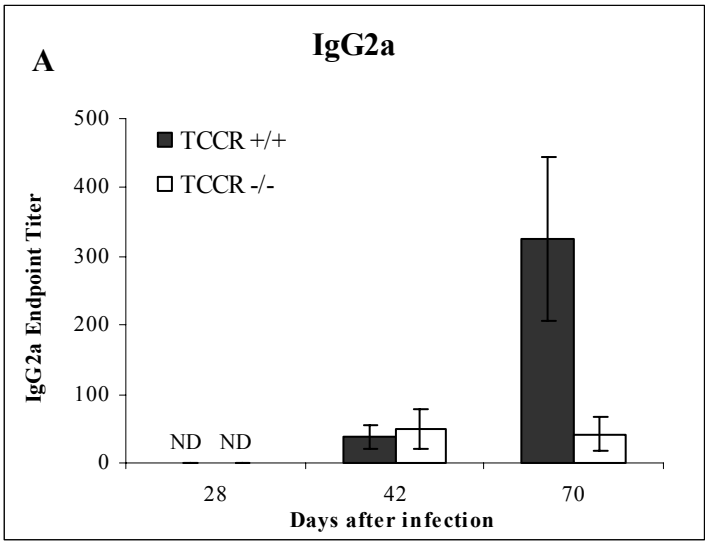

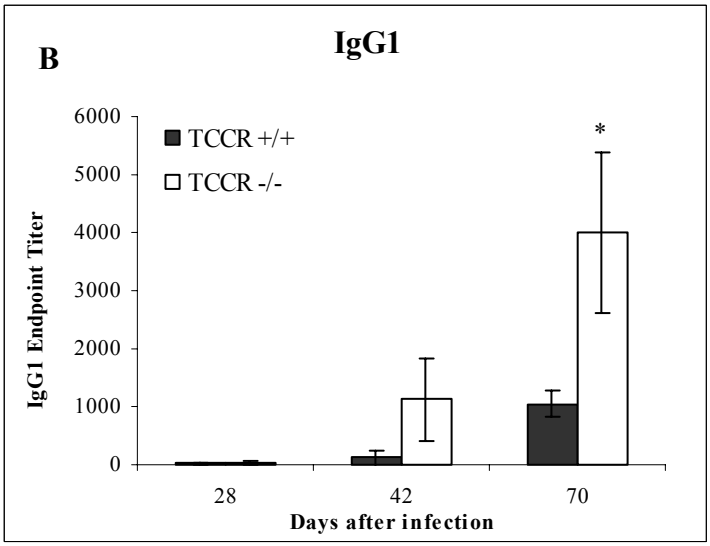

Figure 4

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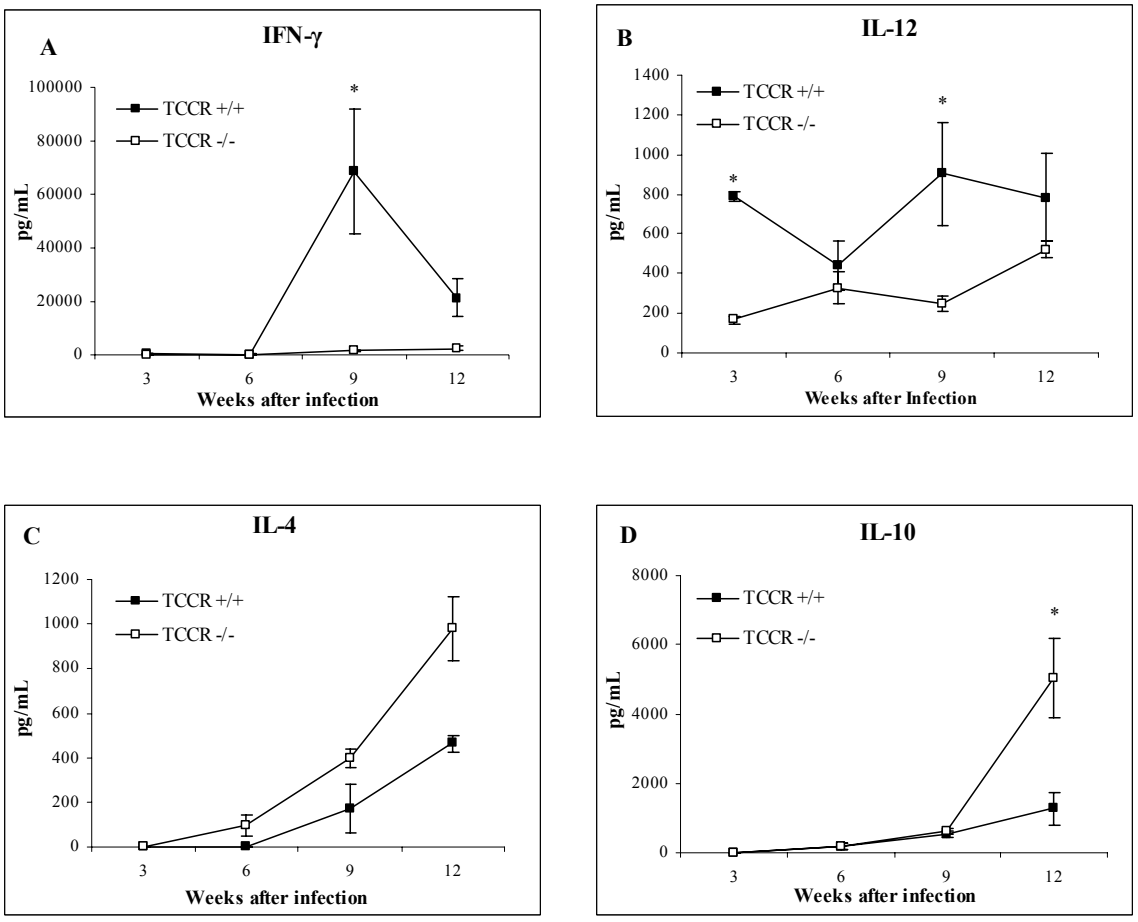

Figure 5

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